Journal of Pharmacological Sciences
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Full Paper

SKI-II, an Inhibitor of Sphingosine Kinase, Ameliorates Antigen-Induced Bronchial Smooth Muscle Hyperresponsiveness, but Not Airway Inflammation, in Mice

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Received August 5, 2010; Accepted September 3, 2010

Abstract. To determine if endogenously generated sphingosine-1-phosphate (S1P) is involved in the development of allergic bronchial asthma, the effects of systemic treatments with SKI-II, a specific inhibitor of sphingosine kinase, on antigen-induced bronchial smooth muscle (BSM) hyperresponsiveness and airway inflammation were examined in mice. Male BALB/c mice were actively sensitized with ovalbumin (OA) antigen and were repeatedly challenged with aerosolized antigen. Animals also received intraperitoneal injections with SKI-II (50 mg/kg) 1 h prior to each antigen challenge. The acetylcholine (ACh)-induced contraction of BSM isolated from the repeatedly antigen-challenged mice was significantly augmented, that is, BSM hyperresponsiveness, as compared with that from the control animals ($P < 0.05$). The BSM hyperresponsiveness induced by antigen exposure was ameliorated by the systemic treatment with SKI-II, whereas the treatments had no effect on BSM responsiveness to ACh in control animals. On the other hand, the systemic treatments with SKI-II had no effect on antigen-induced inflammatory signs, such as increase in cell counts in bronchoalveolar lavage fluids (BALFs) and change in airway histology; upregulation of BALF cytokines, such as interleukin-4 (IL-4) and IL-13; and elevation of total and OA-specific immunoglobulin E (IgE) in sera. These findings suggest that sphingosine kinase inhibitors such as SKI-II have an ability to prevent the development of BSM hyperresponsiveness, but not of allergic airway inflammation. The endogenously generated S1P might be one of the exacerbating factors for the airway hyperresponsiveness, one of the characteristic features of allergic bronchial asthma.

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Keywords: sphingosine kinase, sphingosine-1-phosphate, bronchial smooth muscle, airway hyperresponsiveness, allergic bronchial asthma

Introduction

The dramatic increase in the number of asthma cases over the last decades is of great concern for public health in the world (1). Increased airway narrowing in response to nonspecific stimuli is a characteristic feature of human obstructive diseases, including bronchial asthma. This abnormality is an important sign of the disease, although the pathophysiological variations leading to the hyperresponsiveness are yet unclear. It has been suggested that one of the factors that contribute to the exaggerated airway narrowing in asthmatics is an abnormality of the properties of airway smooth muscle (2, 3). Rapid relief from airway limitation in asthmatic patients by β-stimulant inhalation may also suggest an involvement of augmented airway smooth muscle contraction in the airway obstruction. Thus, it may be important to understand changes in the contractile signaling of airway smooth muscle cells associated with the disease for development of asthma therapy.

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that mediates diverse biological responses, including smooth muscle contraction (4 – 8). Recently, an involvement of S1P in allergic bronchial

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Published online in J-STAGE
doi: 10.1254/jphs.10202FP

asthma has been suggested (9 – 15). Ammit and colleagues (9) firstly demonstrated that S1P levels are elevated in the airways of individuals with asthma after segmental allergen challenge. The finding that S1P can act as a chemotactic agent for eosinophils further suggests an involvement of S1P in pathophysiology of asthma (10). Indeed, inhalation of inhibitors for sphingosine kinase, which produces S1P directly from sphingosine, attenuated antigen-induced airway inflammation in mice (11). In addition, S1P might have an ability to cause airway hyperresponsiveness (AHR) (12 – 15). Contrary to these observations, inhalation of S1P itself or FTY720, an S1P-receptor agonist, prevented antigen-induced airway inflammation and hyperresponsiveness in mice (16). Thus, the role of S1P in the development of asthma and/or AHR is still controversial.

In the present study, to determine if endogenously generated S1P is involved in the development of allergic bronchial asthma, the effects of systemic treatments with 4-[4-(4-chloro-phenyl)-thiazol-2-ylamino]-phenol (SKI-II), a specific inhibitor of sphingosine kinase (17), on antigen-induced bronchial smooth muscle (BSM) hyperresponsiveness and airway inflammation were examined in mice.

Materials and Methods

Animals and treatments

Male BALB/c mice were purchased from Charles River Japan, Inc. (Kanagawa) and housed in a pathogen-free facility. All animal experiments were approved by the Animal Care Committee of the Hoshi University (Tokyo).

Preparation of a murine model of allergic bronchial asthma, which has an in vivo AHR (18), was performed as described previously (19 – 21). In brief, BALB/c mice (8 weeks of age) were actively sensitized by intraperitoneal injections of 8 μg ovalbumin (OA; Seikagaku Co., Tokyo) with 2 mg Imject Alum (Pierce Biotechnology, Inc., Rockfold, IL, USA) on Day 0 and Day 5. The sensitized mice were challenged with aerosolized OA-saline solution (5 mg/mL) for 30 min on Days 12, 16, and 20. The OA aerosol was generated with an ultrasonic nebulizer (Nihon Kohden, Tokyo) and introduced to a Plexiglas chamber box (130 × 200 mm, 100 mm height) in which the mice were placed. SKI-II [50 mg/kg, 25 mg/mL in dimethyl sulfoxide (DMSO)] or its vehicle (DMSO, 20 μL/10-g weight) was administered intraperitoneally 1 h prior to each antigen challenge. The dosage and administration route for SKI-II were decided based on the previous reports (22, 23).

Functional studies

At 24 h after the last OA challenge, mice were sacrificed by exsanguination from the abdominal aorta under urethane (1.6 g/kg, i.p.; Sigma and Aldrich, St. Louis, MO, USA) anesthesia, and the airway tissues under the larynx to lungs were immediately removed. About 3-mm length of the left main bronchus (approximately 0.5-mm diameter) was isolated and the epithelium was removed by gently rubbing with sharp tweezers (24). The resultant tissue ring preparation was then suspended in a 5 mL-organ bath by two stainless-steel wires (0.2-mm diameter) passed through the lumen. For all tissues, one end was fixed to the bottom of the organ bath while the other was connected to a force-displacement transducer (TB-612T, Nihon Kohden) for the measurement of isometric force. A resting tension of 0.5 g was applied. The buffer solution contained modified Krebs-Henseleit solution with the following composition: 118.0 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10.0 mM glucose. The buffer solution was maintained at 37°C and oxygenated with 95% O_2 – 5% CO_2. After the equilibration period, the concentration–response curve to acetylcholine (ACh, final concentration of 10^{-7} – 10^{-3} M) was constructed cumulatively. After the measurement of BSM responsiveness to ACh, the tissues were frequently washed out and re-equilibrated. Then the tissues were depolarized by isotonic high K^+ solution (final concentration of 10 – 90 mM, cumulatively) in the presence of atropine and indomethacin (each at 10^{-6} M).

Cell counts in bronchoalveolar lavage fluids (BALFs)

At 24 h the last OA challenge, mice were sacrificed as described above. After thoracotomy, blood samples were collected from the hearts to obtain sera, and BAL was carried out as previously described (25). The BALF obtained was centrifuged at 500 × *g* for 5 min at room temperature, and the resultant pellet of the centrifuged BALF was resuspended in 500 μL of 10% formaldehyde and incubated for 10 min at room temperature. The cells were washed with PBS and resuspended in 500 μL of PBS. An aliquot of cell suspension was used for cell counts with a hemocytometer.

Quantitation of interleukin-4 (IL-4) and IL-13 levels in BALFs

The resultant supernatant of the centrifuged BALF was used for cytokine analyses. The levels of IL-4 and IL-13 were determined using enzyme-linked immunosorbent assays (ELISAs) with Quantikine™ mouse IL-4 immunoassay (M4000B; R&D Systems, Inc., Minneapolis, MN, USA) and Quantikine™ mouse IL-13 immunoassay (M1300CB; R&D Systems, Inc.), respectively,

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according to the manufacturer's instructions.

Quantitation of immunoglobulin E (IgE) level in serum

Serum was obtained from the blood sample by centrifugation at 3,000 × *g* for 10 min at 4°C. Total IgE and OA-specific IgE in sera were measured by an ELISA system (Bethyl Laboratories, Inc., Montgomery, TX, USA) as described previously (25).

Histological studies

Airways below the main bronchi were fixed in 10% formaldehyde and embedded in Paraplast X-TRA™ paraffin (Fisher HealthCare, Houston, TX, USA). Sections (4 μm) were obtained from blocks and mounted on a silane-coated glass slide, deparaffinized with xylene and graded ethanol, and processed for hematoxylin and eosin staining.

Statistical analyses

All the data were expressed as the mean with S.E.M. Statistical significance of difference was determined by the unpaired Student's *t*-test or two-way analysis of variance (ANOVA) with *post hoc* Bonferroni/Dunn (StatView for Macintosh ver. 5.0; SAS Institute, Inc., Cary, NC, USA). A value of $P < 0.05$ was considered significant.

Results

Effects of systemic treatments with SKI-II on antigen-induced BSM hyperresponsiveness

Figure 1 shows the BSM responsiveness to ACh (Fig. 1: A and B) and high K^+ depolarization (Fig. 1: C and D) in the control and the repeatedly antigen-challenged mice. Animals also received intraperitoneal injection with SKI-II (Fig. 1: B and D), a specific inhibitor of sphingosine kinase (17), or its vehicle (Fig. 1: A and C) as described in Materials and Methods. Application of ACh ($10^{-7} – 10^{-3}$ M) to isolated BSMs elicited a concentration-dependent contraction in all animals used (Fig. 1: A and B). The contractile response to ACh of BSM from the repeatedly antigen-challenged mice was significantly augmented, that is, BSM hyperresponsiveness, as compared with that from the control animals (Fig. 1A; $P < 0.05$, by two-way ANOVA). On the other hand, no significant change in the contractile response induced by high K^+ depolarization was observed between the groups (Fig. 1C). These findings are consistent with our previous reports (20, 21, 24) that demonstrate an augmented agonist-induced Ca^{2+} sensitization of contraction in the repeatedly antigen-challenged mice. The results also indicate that the vehicle used had no effect on the BSM hyperresponsiveness induced by antigen exposure.

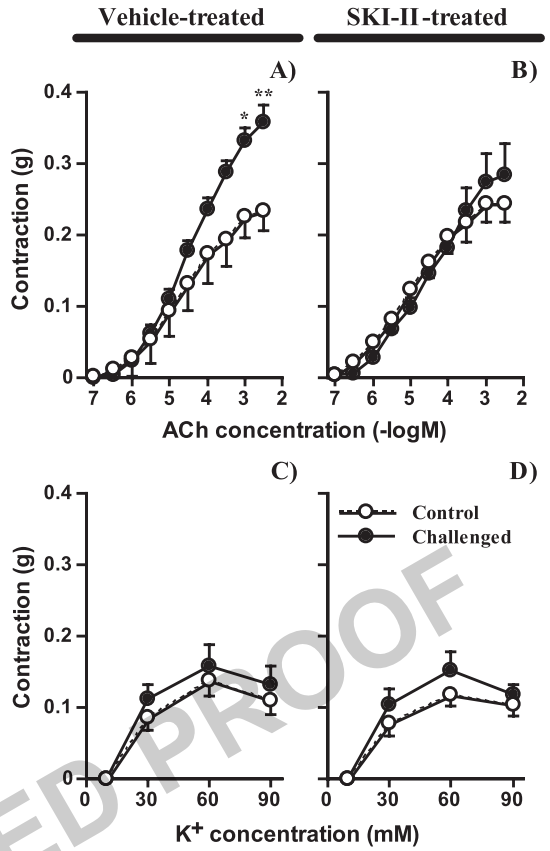

Fig. 1. Effects of systemic treatment with SKI-II, an inhibitor of sphingosine kinase, on bronchial smooth muscle (BSM) hyperresponsiveness to acetylcholine (ACh) in repeatedly antigen-challenged mice. The contractile responsiveness to ACh (A and B) and high K^+ (in the presence of atropine and indomethacin, both at 10^{-6} M; C and D) were measured in BSMs isolated from the sensitized control (Control: open circles) and the repeatedly antigen-challenged mice (Challenged: closed circles). Animals also received intraperitoneal injections with SKI-II (B and D) or its vehicle (A and C) as described in Materials and Methods. Each point represents the mean with S.E.M. from 6 different animals. *$P < 0.05$ and **$P < 0.001$ vs. Control by Bonferroni/Dunn's test. Note that the BSM hyperresponsiveness to ACh observed in the antigen-challenged group (A) was abolished by the systemic treatment with SKI-II (B). Neither antigen exposure nor SKI-II had significant effect on the BSM responsiveness to K^+ depolarization (C and D).

In the control animals, the systemic SKI-II treatments had no effect on the contractility of BSM to ACh (vehicle-treated control in Fig. 1A vs. SKI-II–treated control in Fig. 1B) or K^+ depolarization (vehicle-treated control in Fig. 1C vs. SKI-II–treated control in Fig. 1D). However, the augmented ACh-induced contraction, that is, BSM hyperresponsiveness, observed in the repeatedly antigen-challenged mice was significantly inhibited by the systemic treatments with SKI-II (vehicle-treated, challenged group in Fig. 1A vs. SKI-II–treated, challenged in Fig. 1B; $P < 0.05$ by two-way ANOVA). The BSM respon-

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siveness to ACh of the antigen-challenged animals returned to the control level almost completely; no significant difference was observed between the repeatedly antigen-challenged and control groups in the SKI-I–treated animals (Fig. 1B).

Effects of systemic treatments with SKI-II on antigen-induced airway inflammation

To determine the effects of inhibition of sphingosine kinases by SKI-II on the changes in airway biology induced by repeated antigen exposure, we recorded total cell counts in BALFs (Fig. 2A) and performed histological examinations (Fig. 3). Total cell counts in BALFs of the repeatedly antigen-challenged mice were significantly increased as compared with those of the control group (Fig. 2A, vehicle-Sens vs. vehicle-Chal group; $P < 0.01$). Diff-Quik staining of the cells revealed that most of the increased cells were eosinophils in this animal model of airway inflammation (21, 26, 27). The systemic treatment with SKI-II had no effect on the increase in total cells induced by antigen challenge (Fig. 2A, vehicle-Chal vs. SKI-II-Chal group). Histological examinations also revealed a marked inflammation of the lungs in the repeatedly antigen-challenged mice: many inflammatory cells were infiltrated into the subepithelial and smooth muscle layers of intrapulmonary bronchi by the antigen exposure (Fig. 3B). Similar histological changes were also observed in the SKI-II–treated, antigen-challenged animals (Fig. 3D).

As shown in Fig. 2, B and C, the levels of IL-4 and IL-13 in BALFs of repeatedly antigen-challenged mice (vehicle-Chal groups) were significantly increased as compared with those of sensitized control animals (vehicle-Sens groups). The systemic treatment with SKI-II had no effect on the levels of these cytokines: no significant difference was observed between the vehicle-Chal and SKI-II-Chal groups in any parameter measured.

Figure 2, D and E, shows the levels of total and OA-specific IgE in sera of mice, respectively. Both the levels of total and OA-specific IgE were significantly increased when animals were repeatedly challenged with OA antigen (both vehicle-Chal groups: $P < 0.05$ vs. respective vehicle-Sens groups). The increase in the level of OA-specific IgE was significantly augmented by the systemic

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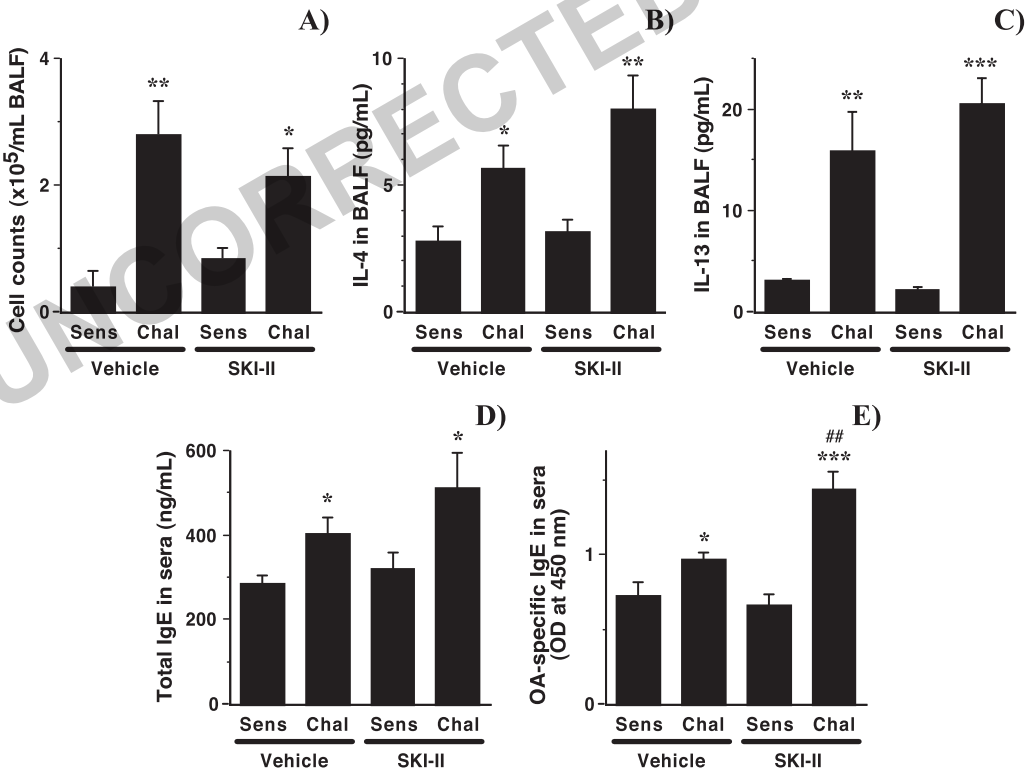

Fig. 2. Effects of systemic treatment with SKI-II, an inhibitor of sphingosine kinase, on the levels of inflammatory cells (A), interleukin-4 (IL-4; B), and IL-13 (C) in bronchoalveolar lavage fluid (BALF), and total (D) and ovalbumin (OA)-specific IgE (E) in serum in sensitized control (Sens) and repeatedly antigen-challenged (Chal) mice. Animals also received intraperitoneal injections with SKI-II or its vehicle as described in Materials and Methods. Each column represents the mean ± S.E.M. from 6 independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. respective Sens group and $^{\#\#}P < 0.01$ vs. Vehicle-Chal group by Bonferroni/Dunn's test.

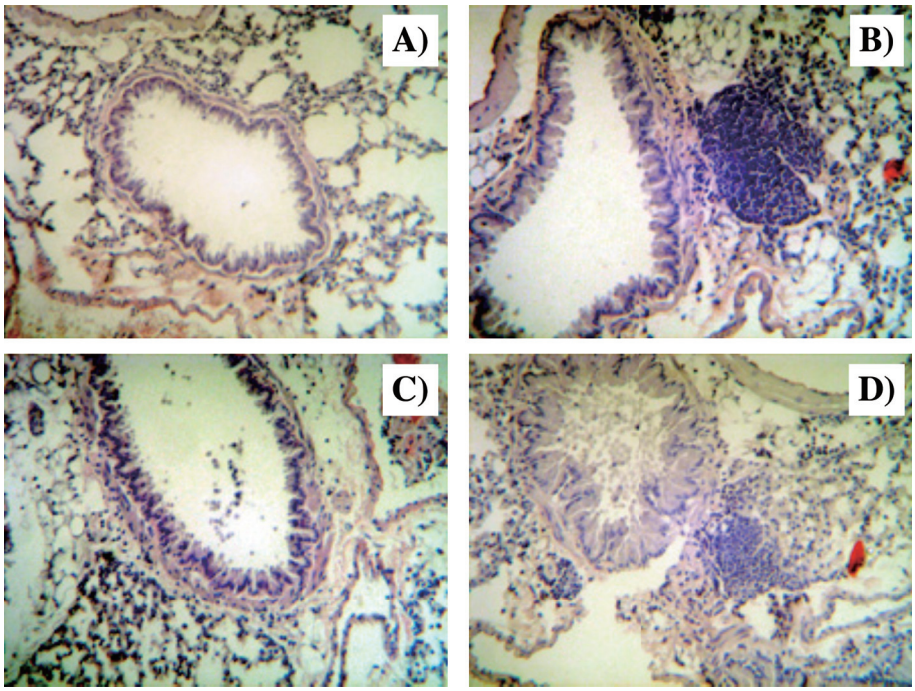

Fig. 3. Effects of systemic treatment with SKI-II, an inhibitor of sphingosine kinase, on airway inflammation induced by repeated antigen challenge in mice. The sensitized control (A and C) and repeatedly antigen-challenged animals (B and D) also received intraperitoneal injections with SKI-II (C and D) or its vehicle (A and B) as described in Materials and Methods. Sections (4 μm) of formalin-fixed tissues were stained with hematoxylin and eosin before examination by light microscopy. Original magnification: ×80. Many inflammatory cells were infiltrated into the subepithelial and smooth muscle layers of intrapulmonary bronchi by the repeated antigen exposure (B). The systemic treatments with SKI-II had no effect on the infiltration of inflammatory cells (D).

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treatments with SKI-II (Fig. 2E, vehicle-Sens vs. SKI-II-Chal group; $P < 0.01$), whereas no significant difference in total IgE level was observed between the groups (Fig. 2D, vehicle-Sens vs. SKI-II-Chal group).

Discussion

The current study clearly showed that in a mouse model of allergic bronchial asthma, systemic treatments with SKI-II ameliorated the augmented ACh-mediated BSM contraction, that is, BSM hyperresponsiveness, induced by repeated antigen exposure, whereas it did not affect the induction of airway inflammation. Because SKI-II is known as a specific inhibitor of sphingosine kinase (17), a main enzyme that mediates S1P biosynthesis (28), the results also suggest that endogenously generated S1P is involved in the development of BSM hyperresponsiveness, one of the causes of AHR in asthmatics.

Recently, an involvement of S1P in the pathogenesis of allergic bronchial asthma has been suggested by the fact that S1P levels are elevated in the airways of asthmatics (9). S1P is synthesized by sphingosine kinases, which phosphorylate sphingosine to generate S1P, and is degraded by S1P phosphatases, lipid phosphate phosphatases, or S1P lyase (28). Various types of cells in the airways are known to produce S1P. In mast cells, activation of the FcεRI receptor activated sphingosine kinases, and both the decrease in sphingosine and the increase in S1P are thought to contribute to the critical increase in intracellular Ca^{2+}, cytokine production, and degranulation (29). In mouse peripheral airways, expression of sphingosine kinase was demonstrated in the airway smooth muscles, and the activation of sphingosine kinase was mediated by the activation of M_2 muscarinic receptor (30). Furthermore, it has also been suggested that sphingosine kinase is activated by various stimuli, such as TNF-α (31, 32), bradykinin (33), and the protein kinase C activators (34). It is thus possible that sphingosine kinase is activated in the airways during the initiation and/or progression of allergic bronchial asthma.

AHR is a common feature of patients with allergic bronchial asthma. It has been suggested that one of the factors that contribute to the AHR in asthmatics is an abnormality of the nature of airway smooth muscles (2, 3). The airway smooth muscle is the main structure of airway walls and plays a major role in the contraction of the airways. The excessive contraction of airway smooth muscles may be one of the crucial factors that directly cause asthma symptoms such as the AHR. Consistent with our previous reports (19 – 24), an increased responsiveness of the isolated BSM to ACh, but not high-K^+ depolarization, was observed in mice that were actively sensitized and repeatedly challenged with OA antigen (Fig. 1). The current study revealed that the antigen-induced BSM hyperresponsiveness was inhibited by systemic treatments with SKI-II (Fig. 1). Similarly, the in vivo AHR induced by antigen exposure in mice was inhibited by pretreatment with other kinds of sphingosine kinase inhibitors, DL-threo-dihydrosphingosine (DTD)

(12), *N*,*N*-dimethylsphingosine (DMS) (11, 35), and 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole (SK-I) (11). The in vivo AHR induced by antigen challenge was also attenuated in the sphingosine kinase-1 knockout mice (14). On the other hand, the exogenously administered S1P itself could reproduce the AHR in naive mice (12 – 15). These findings indicate that endogenously generated S1P via an activation of sphingosine kinase is involved in the development of the AHR. In contrast, our recent study revealed that in BSMs of the antigen-induced AHR mice, the expression level of S1P receptor 2 (S1PR2) was markedly decreased, resulting in a loss of the S1P-mediated contraction (36). Thus, S1P itself does not contribute to bronchoconstriction in an asthma attack, but may be involved in early stage of the AHR development. Although S1P itself was not measured in the present study, the observations that S1PR2 was downregulated (36) and that mRNA for sphingosine kinase was upregulated (our personal observations) in the airways of the repeatedly antigen-challenged mice suggest that S1P level might be increased in the airways of mouse experimental asthma.

Allergic airway inflammation is also a sign of asthma. It has been reported that sphingosine kinase inhibitors such as DMS (11, 35) and SK-I (11) could attenuate antigen-induced airway inflammation. However, in the present study, the SKI-II treatments had no significant effect on the increase in BAL cells (Fig. 2A) and the histological changes in lungs (Fig. 3) induced by antigen exposure. Although the discrepancy cannot be explained accurately here, the extent of inhibition of airway inflammation may depend on the inhibitor used. Indeed, Nishiuma and colleagues (11) reported that the extent of inhibition of airway inflammation caused by DMS was higher than that caused by SK-I, while the extent of inhibition of AHR caused by SK-I was higher. Unexpectedly, the current study also revealed that SKI-II caused an augmentation of antigen-induced increase in OA-specific IgE in sera (Fig. 2E), whereas Kurashima and colleagues (37) reported that systemic treatment with FTY720 had no effect on IgE production in allergic mice. Further studies are needed clarify the exact role of sphingosine kinase on the development of airway inflammation and hyperresponsiveness.

In conclusion, sphingosine kinase inhibitors such as SKI-II might have an ability to prevent the development of BSM hyperresponsiveness, but not of allergic airway inflammation. The endogenously generated S1P might be one of the exacerbating factors for AHR, one of the characteristic features of allergic bronchial asthma.

Acknowledgments

We thank Ms. Yuka Narushima and Ms. Tomoko Minemura for their technical assistance.

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